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GENETIC MONITORING SYSTEMS FOR ENVIRONMENTAL FATE ASSESSMENT OF MICROBIAL WEED CONTROL AGENTS: PART II

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Title: Genetic Monitoring Systems for Environmental Fate Assessment of Microbial Weed Control Agents, Part II: Testing the genetic tools developed for monitoring biocontrol fungi in plant tissues and soil samples.

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Abstract/Summary:
Before registering a microbial weed control product, we need to understand how the organism survives and disperses in the environment. This study determined the ability of a biocontrol fungus, applied by broadcasting granules, to colonize plants, and move and persist in soil. Resistant and susceptible crop species, and weed hosts were colonized, although fungal isolations were low and declined with time. Using a highly specific DNA probe, the fungus was detected in grass roots from 67% of the inoculated plots for up to 9 weeks after application, but was rarely detected (16%) after 38 weeks. The fungus was isolated by soil dilution plating for 30 days after application, but the number of colonies declined with time. The DNA probe identified the presence of the fungus in the top 0-3 inches of soil in 92% of inoculated plots for up to 9 weeks, but the frequency declined to 40% after 38 weeks. At lower soil depths (4-6 inches) the fungus was not detected until after 38 weeks. Less than 10% of the soil samples taken at 12 and 24 inches from the plot edges and untreated plots had the fungus present, which resulted from contamination during application.

Executive Summary:

When applying a microbial pest control product for biological control in the field, it is important to know that the strain being released reaches the intended target and does not cause deleterious effects by moving away from the site of application or by persisting for too long. Monitoring the strain becomes difficult when it is applied to soil because many microbes living in that environment may be phenotypically similar to the released strain. Traditional microbiological methods for isolation and characterization may be used, but the detection process may be greatly aided by genetic tools developed for the specific biocontrol strains. Genetic tools have been developed for the identification of specific isolates of a biocontrol fungus that controls Canada thistle and other important weed pests, such as dandelion, chickweed, and scentless chamomile. The objective of this project was to use a combination of traditional microbiological and molecular genetic methods to monitor the colonization of plant tissues, the movement in soil, and the persistence in soil by the biocontrol fungus.

Using microbiological methods, the research showed that the biocontrol fungus colonized tissues of both resistant (barley) and susceptible (lentil) crop species and a susceptible weed host (Canada thistle) when these plants came into contact with the fungus after a broadcast application to the soil before plant emergence. Based on the

literature, this fungal species is described as having a weak saprophytic nature, primarily isolated from woody tissues with incidental occurrences on herbaceous plants throughout the world. The low frequency of isolation from tissues (<25%) in this study and the decline in isolation frequency over time supports the idea of its weakly pathogenic nature as presented in the literature. The fungus was also isolated from plant tissues of barley and lentil grown in untreated soil, which suggested that naturally-occurring contamination of seed and soil may be expected. Short-term greenhouse studies indicated that the biocontrol fungus persisted in soil for at least 30 days, but the number of viable colonies significantly declined to nearly zero during that time. Re-planting a susceptible weed host (dandelion) into the 30-day old inoculated soil showed residual weed control activity despite the low number of viable colonies.

A DNA probe was developed, which was specific for several isolates of the biocontrol fungus. The probe was used to detect the presence of the fungal DNA in soil and plant root samples. The probe was sensitive enough to detect the presence of the fungus at lowest rate of field application. On average, less than 5% (range of 0-10%, n=106) of the untreated control plots had the fungus present in the soil and on grass roots. About 45% of all treated plots had the fungus detected (range from 0-92%, n=264). For up to 9 weeks after inoculation, the fungus was detected on grass roots in 67% of the plots, but this declined to 16% after 38 weeks. In soil, the fungus was detected in the 0-3 inch deep soil profile in 92% of the plots tested, but declined to 40% of the plots after 38 weeks. Outside of the plot area, at 12 and 24 inches from the plot margin, the fungus was occasionally detected in some soil samples (11% of plots), which was similar to the random detection of the fungus in the untreated controls (5%). The contamination in the margin areas only occurred in one direction (only on the south edges) and only with plots that had higher rates of application than 250 g/m^2. The contamination most probably occurred from applicator error and from windy conditions at the time of inoculum application. There was some indication that that the fungus moved downward in the soil profile over time as it was not detected at the 4-6 inch depth for up to 9 weeks after inoculation, but was found in 75% of the plots (n = 4) after 38 weeks, however the sample size was too small to make definitive conclusions.

In this study, the biocontrol fungus displayed characteristics that are desirable in a microbial pest control product. Its ability to colonize both resistant and susceptible plant tissues indicates that it has a saprophytic rather than pathogenic nature. Declining frequency of isolation and detection by molecular methods from plant tissues and soil over time provides evidence that the fungus does not multiply, reproduce, or persist on target and non-target host plants, and in soil. In the soil, the fungal populations declined by nearly 50% after 38 weeks. At these levels, the biocontrol fungus is unable to cause damage to susceptible host plants because a related study showed that susceptible crops (such as pea and sunflower) may be sown into soils previously treated with the biocontrol fungus (one year following application to the soil) without developing chlorosis or other effects on emergence and plant growth. It is also unlikely that the biocontrol fungus can move any significant distance horizontally in soil. The fungus was detected at similar levels in the plot margins as in the untreated control plots, and the frequency of detection in these areas declined with time, thereby reducing the risk of spreading the fungus to

neighboring non-target plants. There is some evidence that the biocontrol fungus may move vertically in the soil profile over the course of a year. However, most fungi cannot survive for long periods in deeper soil depths due to less oxygen. Further studies may need to be done to determine survival over time at deeper soil depths. These features greatly reduce the risk of the fungus spreading and persisting in the environment.

Two different approaches were used in this study. Similar observations obtained by both methods helped to validate the results, despite some limitations with the methods. The traditional microbiological techniques for isolation of fungi from plants and soil detected fungal viability, as well as relative population numbers. The limitations were in developing a semi-selective medium for identification of the correct fungal species; note it was not possible to know whether the colonies were from the released strain or a natural wildtype contaminant. Using the specific DNA probe, identification of the exact strain was assured, but it was not known whether the DNA came from living or dead propagules. If no fungal DNA was detected then it could result from its absence in the plot, degradation of the DNA by other microorganisms, or possibly failure in the technique. Extracting clean samples of fungal DNA from soil is difficult and soil samples with higher amounts of organic matter can affect the quality of the DNA.

In conclusion, the biocontrol fungus had limited impact on the environment. The effects were mostly limited to the season of application and the site of placement. The greatest effects were observed at the time of application and for up to 9 weeks. After that time the fungal populations declined, so carryover from season to season was significantly reduced. Care may need to be exercised during application to prevent drift onto to emerging non-target plants nearby the site of application if rates greater than 63 g/m^2 are used.

Technical Report:

Weeds can be controlled by an inundative application of naturally occurring fungi, whereby a large quantity of the organism is applied directly to a weed infested area in order to achieve rapid control. When testing biocontrol fungi in the field, it is important to know that the released strain is reaching the intended target weed and is not having deleterious effects on non-target plant species or the environment. Before being able to register a fungus as a microbial weed control product, we need to provide evidence on the survival and dispersal of the fungus in the environment. This becomes difficult when the biocontrol fungus is applied to the soil where many thousands of other organisms are also present. Traditional microbiological methods may be used to isolate the fungus, but it is difficult to know whether it is the actual biological control strain or a phenotypically related one. Genetic tools have been developed to aid in the detection of specific isolates of fungi being developed for biological control of Canada thistle and other important weed pests, such as dandelion, chickweed, and scentless chamomile. The objective of this project was to use a combination of traditional microbiological and molecular genetic methods to monitor the colonization of plant tissues, movement in soil environments, and persistence in soil by selected isolates of the biocontrol fungus.

I. Detecting Plant Colonization and Fungal Persistence by Microbiological Methods

A) Plant Colonization

A study was conducted in the greenhouse to determine the ability of the biocontrol fungus to colonize tissues of weed hosts and non-target crops species, which were grown in soil treated with the fungus. It was hypothesized that biocontrol agent would colonize the roots of the host weeds and susceptible crops, but not resistant crops.

Fungal inoculum was prepared by growing the biocontrol fungus on grains in jars for 2 weeks, air-drying the colonized grain, and then grinding it. The plant species being evaluated were Canada thistle as the target weed, barley cv. Tercel as a resistant crop plant, and lentil cv. Laird as a susceptible crop plant. Planting medium was used to fill 4-inch pots ¾ full and then the surface was sprinkled with 5 g of fungal inoculum. Seeds of barley and lentil (5/pot) and root pieces of Canada thistle (2-2inch pieces/pot) were placed on the inoculum, covered with planting medium, and taken to the greenhouse for the duration of the experiment. Plants grown in pots without fungal inoculum were used as the untreated control.

Plants were harvested from pots at 10, 20, and 30 days after seeding. The root-end of plants were placed in a beaker with 50 ml sterile water and shaken to collect the rhizosphere-soil wash for a study on the persistence of the biocontrol fungus in soil (see section below). The plants were then placed under running water for 45 minutes and surface sterilized before plating the plant tissues on plates of potato dextrose or cherry juice agar with antibiotics. The plant tissues consisted of 6 leaf and 6 root segments per plant (each segment being 2 cm in length), and the plant crown (i.e. tissue at soil-air interface). The plated tissues were incubated at room temperature with natural light for 5-10 days. The plates were rated for the number of colonies that appeared to have growth similar to the biocontrol fungus. Two experiments were conducted in a completely randomized design with 3-6 replications. A square root transformation was applied to the data before being analyzed by analysis of variance and tests of least significant difference (LSD at $P<0.05$) for the main effects. Untransformed means are presented.

The frequency of fungal isolation from plant tissues using microbiological methods was analyzed separately for the two trials due to significant differences in treatment effects and interactions (Table 1). In Trial 1, the frequency of isolation changed with time, whereas in Trial 2 all treatment effects were significant. In both trials, there were interactions among treatment of the soil with the biocontrol fungus and the host plants, and time after seeding.

The overall mean frequency of isolation from plant tissues grown in treated plots was low, being 25% in Trial 1 and 18% in Trial 2 (Table 2). In Trial 1, the biocontrol fungus was also isolated at the rate of 12% from some plants grown in untreated control plots, but none was isolated in Trial 2. The fungus was found in lentil leaves and roots grown in control plots at 10 days and in barley leaves after 30 days.

In Trial 1, the biocontrol fungus was isolated more frequently from the susceptible host plants, Canada thistle and lentil, at both 10 and 30 days after seeding (Table 2). In lentil, the frequency of isolation decreased with time. The fungus was only isolated from barley after 30 days. There were no significant differences in the isolation frequency from leaf, root, and crown tissues. In Trial 2, the fungus was isolated in similar frequencies from barley and lentil, but none was isolated from Canada thistle. It was

isolated more often from roots (14%) and leaves (12%) as compared to crown tissues (2%). The highest frequency of fungal isolation occurred from plant samples taken at 10 days after seeding; lower frequencies of isolation occurred at both 20 and 30 days.

The data showed that the biocontrol fungus colonized both resistant and susceptible crop species and a susceptible weed host when applied as a pre-emergent granular application to the soil. This conclusion is different than the original hypothesis, which indicated that resistant plant species would not be colonized. Based on the literature, this fungal species is described as having a weak saprophytic nature, primarily isolated from woody tissues with incidental occurrences on herbaceous plants. The low frequency of isolation (< 25%) supported the ideas presented in the literature that this fungus is more opportunistic and not pathogenic. The fungus has a worldwide distribution and naturally lives in the soil and on seed, so contamination of seed and soil may occur in supposedly untreated soils. Also the frequency of fungal isolation from plants had a tendency to decline with time. This is a positive feature in a microbial pest control product because the fungus will not rapidly reproduce and increase the quantity of inoculum on target and non-target plants in the environment. Less inoculum reduces the risk of disease spread.

B) Fungal Persistence in Soil

A greenhouse study was conducted to evaluate the persistence of the biocontrol fungus in soil over a 30 day period from the time of application (see Plant Colonization for methods). The rhizosphere-soil, collected in a water wash from roots of barley, lentil, and Canada thistle at 10- 30 days after seeding, was dilution-plated on to potato dextrose or cherry juice agar with antibiotics to determine the number of fungal colonies present with growth similar to the biocontrol fungus. At 30 days, all remaining plants were removed and dandelion seeds (20 seeds/pot) were planted into the residual soil in each pot in the greenhouse. The emergence and % chlorosis of the dandelions were recorded 10 and 20 days after seeding to determine if there were residual effects of the previous treatments with host plants and fungal inoculum. In Trial 2 before planting the weed seeds, a core of soil was taken from each pot for dilution plating. Data were analyzed by analysis of variance and least significant difference tests at $P<0.05$ for main effects and the means for fungal isolations were calculated over all dilutions.

Analyses of variance of isolations from rhizosphere soil were similar in both Trial 1 and 2 (Table 3). The biocontrol fungus was infrequently isolated from untreated soils (Table 4). The isolation frequency in all host plants declined over time. The biocontrol fungus was more often isolated from the rhizosphere of lentil than Canada thistle (Trial 1 only) and barley. In Trial 2, the fungus was not isolated from the rhizosphere from Canada thistle. The soil core samples taken in Trial 2 also showed that no biocontrol fungus was present in the soils that had Canada thistle, but it was present at low levels in the soils that had barley and lentil (Table 5).

The residual effect of treating the soil with the biocontrol agent and the impact of the previous host crop had significant effects on the emergence and chlorosis of dandelion (a susceptible weed). The two trials were analyzed separately due to different responses in each (Table 6). In Trial 1, dandelion emergence was lower and chlorosis was higher in all soils previously treated with the biocontrol fungus as compared to the untreated soils (Table 7). In Trial 2, similar observations were noted for soils previously

planted to barley and lentil, but not with Canada thistle (i.e., no reduction in emergence and no chlorosis).

The results from the Plant Colonization and Peristence studies with Canada thistle in Trial 2 were not expected. It suggest that there was an experimental error when the test was being set up, which probably resulted from the application of noninfested ground grain being applied to soil instead of the biocontrol fungus. In other studies, noninfested grain is used as a control, but has the same appearance as the ground grain infested with the fungus.

The results from the studies on persistence indicated that in the short term, the biocontrol fungus persists in the soil for at least 30 days, but the number of viable propagules significantly declined during this time. There was residual weed control activity occurring 30 days after the biocontrol fungus was applied to the soil, which may result in season-long weed control.

II. Detecting Plant Colonization, Fungal Persistence, and Movement in Soil by Molecular Genetic Methods

A) Detection of the Biocontrol Fungus in Plants and Soil with a DNA Probe

The development of a reliable specific-DNA probe was essential for this study. Some difficulties had been encountered, which needed to be resolved before beginning the study. The probe insert had been sequenced but primers could not be found that would detect the fungal species using PCR (polymerase chain reaction) technology. The probe was sequenced again and the results showed missing fragments at both ends of the probe, and also missing nucleotides in mid-sequence. Based on the known information of vector sequence and the sequencing results of the insert, the complete sequence of probe p1-6 has been obtained. It is a 1370-base probe (insert) cloned in pBluscript KS II. Two sets of primers for PCR detection were developed for this probe insert product. Protocols were optimized for monitoring the target fungi in the plant root and soil samples.

Plants were carefully removed from the soil to keep the roots intact. The plant roots were placed in a beaker of water and shaken for 30 min to remove attached soil, and then rinsed under tap water until the roots were visibly free of soil particles. The samples were shaken again in tap water for 30 min and then rinsed with tap water. The final rinse used distilled water and then roots were wrapped in paper towel to remove excess moisture. Plant DNA was extracted using a crude plant DNA extraction protocol based on Edwards et al. (Nucleic Acids Research 1991, 1996). When necessary the DNA samples were purified through an RNase-A treatment followed by a phenol-chloroform extraction procedure.

Soil samples about 5-50 g each were collected. For time course investigations, only upper-core soil (0-3") samples were taken for molecular analysis. For spatial monitoring, upper-core soil, lower-core soil (4-6") and outside-plot soil samples (i.e., 12" and 24" apart from the plot edge) were collected for analyses. Fine soil particles of less than 1 mm, without obvious plant debris, were used for DNA isolation. Several different DNA isolation methods, including the above mentioned plant DNA isolation method and bead mill homogenization methods based on the description of Kuske et al. (1998) were tried. The quality of DNA samples obtained by all these methods was not good enough for use with PCR, most probably due to humic acid inhibitors in the soil. Finally, high

quality soil DNA was obtained with the UltraClean™ Soil DNA Kit (MO BIO Laboratories, Inc.).

A preliminary study was conducted in the greenhouse to determine PCR detection sensitivity of the biocontrol fungus using the probe with DNA extracted from plant roots and soil. Using the methods previously described for inoculum preparation and application to pots, grass seed (cv. Overseeding Mixture) was sown as a resistant host in field soil at the rate of 0.11g per 10 cm^2 pot. Seven doses of fungal inoculum were applied at the rates of 0, 4, 8, 16, 32, 63, and 125 g/m^2. It is anticipated that 8 or 16 g/m^2 will be the commercial rate of application. After three weeks of growth, plant roots and soil were sampled for DNA extraction. The experimental design was a randomized complete block with 4 replications, but only 2 replications were used for DNA analyses.

The biocontrol fungus was not detected on roots or in soil of the untreated control plots (Table 8). It was detected in all soil samples at all rates of application. The fungus was not found on the roots of plants grown in soil treated with 4 g/m^2 of the biocontrol fungus, however it was detected at all other rates of application. These results indicate that the probe was highly sensitive in detecting low amounts of the biocontrol fungus.

B) Plant Colonization and Fungal Persistence on Roots

Several field trials were used for molecular monitoring of the biocontrol fungus in both plants and soil. The same studies were used to monitor colonization of grass roots by the biocontrol fungus over time and also the movement and persistence of the biocontrol fungus in soil. The field experiments were conducted as randomized complete block designs with 4 replications, but only 2-3 reps were used for molecular monitoring. The experiments were named as:

1) Dose Effect 2002 applied one isolate of the biocontrol fungus to soil at different doses ranging from 0-1000g/m^2 on June 24, 2003,
2) Isolate Effect 2002 applied 3 different isolates of the biocontrol fungus to the soil on June 24, 2003,
3) Dose Effect 2002 applied different production batches of one isolate of the biocontrol fungus to the soil at doses ranging from 0-1000g/m^2 on August 28, 2002 for sampling over time, and
4) Dose Effect 2001 applied one isolate of the biocontrol fungus to the soil at two doses (0 and 1000g/m^2) on May 28, 2001 for sampling within the season of application (September 9, 2001) and the following year (July 24, 2002).

Grass roots were colonized by the biocontrol fungus in 3 of 4 experiments. One month after application of the fungus to soil, DNA of the biocontrol fungus was extracted from the grass roots at 1000 and 63 g/m^2, but not at 250 g/m^2 (Table 9.1). About 3 months after inoculation, the biocontrol fungus was detected at all rates of application, but the bands were considerably weaker than at the earlier sampling time (Table 9.2). About 10 months after inoculation, the biocontrol fungus was only detected in one replicate (Table 9.3). Similar responses in root colonization over time were observed with the 3 strains of the biocontrol control fungus (Table 10). Intensive weekly and biweekly monitoring for two months after inoculation showed roots were colonized as they were being established, since the fungus was detected at 2 weeks after seeding (Table 11).

These roots continued to be colonized for up to 9 weeks. After 38 weeks, the fungus was not longer detected at the 250 g/m^2 and intermittently at the 500 g/m^2 rates. In the fourth, experiment, which was conducted as a dose experiment in 2001, there was no biocontrol fungus detected in root sample taken about 3 and 14 months after inoculation (Table 12). Based on the four field experiments, 67% of root samples taken from inoculated plots were colonized by the biocontrol fungus for up to 9 weeks after inoculation, but by 38 weeks or longer colonization decreased to 16%. The untreated control plots had about 9% of the plots contaminated with the fungus, but detection decreased to zero by 38 weeks.

The biocontrol fungus was able to colonize roots of a resistant host during the growing season when the product was applied. The weak banding patterns that were produced with later sampling times indicated that the fungal population was not as intense as when first sampled and the population has declined. Contamination of a few control plots was likely due to applicator error, which is further explained below. There are some limitations with using PCR detection. Although a positive response indicates the real presence of the fungus, a negative response may result for more than one reason. For example, a negative result may indicate the true absence of the fungus or it may indicate poor sampling or failure in the reliability of the technique. The latter may occur is the sample taken contained a higher proportion of organic matter and humic acid, which can result in poor quality extractions of the DNA. It was also suspected that the storage of moist soil samples in a freezer for 13 months before DNA analyses resulted in DNA degradation. These reasons may explain the discrepancies among replicates.

C) Fungal Movement and Persistence in Soil

This study evaluated the ability of the biocontrol fungus to move laterally and vertically from an area of application in the soil over time. All methods, experiments, and treatments were previously described.

Using the DNA probe, the biocontrol fungus was detected in most of the soil samples taken from the treated areas, but only occasionally from untreated soils. At the 0-3 inch depth of soil and up to 9 weeks after inoculation, 92% of all treated plots had the biocontrol fungus detected , whereas only 4% of untreated plots indicated that the fungus was present (Tables 9-12). After 38 weeks, detection of the fungus in treated plots decreased to 40%. At the 4-6 inch depth of soil, the biocontrol agent was not detected for up to 9 weeks, but it was found at this depth after 38 weeks (Table 11). There was no biocontrol fungus detected in the control plots at 4-6 inches deep.

The biocontrol fungus was detected at rates of application between 63-100 g/m^2 for up to 14 months after application (Table 9, Table 11, Table 12). However, after 38 weeks, the fungus was detected less frequently and by 14 months the bands were very weak. These two observations indicate that the populations had declined with time. The DNA probe was able to detect the presence of three strains of the biocontrol fungus and the frequency of detection decreased with time (Table10).

The biocontrol fungus was occasionally detected in soil cores taken at 12 and 24 inches (in 6% and 16% of plots, respectively) from the treated plot margins in 3 of 4 experiments (Tables 9-12). However, the frequency of occurrence was similar to that found in the control plots (5% of plots at both 12 and 24 inches). The bands were often weak (Table 9 and 10). It was also observed that the fungus was only detected on the southern side of the plots. It is probable that this effect was caused by southbound winds

at the time of applying the formulation or applicator error from using the south edge of plots as a preparatory area when applying inoculum, since the fungus was never detected from the north side of the plots (Note: only north and south edges were sampled.)

The biocontrol fungus was detected from soil samples in areas where inoculum was applied. Over time, the fungal population declined in the soil, suggesting there will not be a buildup of inoculum on plant residues. The fungus was relatively stationary and did not naturally move horizontal distances in the soil profile, thus reducing risk to neighboring non-target plants. However, displacement may occur by man's error if care is not taken at application. There was also some movement vertically in the soil profile. However, at these depths there is often less microbial activity because oxygen becomes limiting, thereby creating an environment that is not suitable for fungal growth.

III. Conclusions

In this study, the biocontrol fungus displayed characteristics that are desirable in a microbial pest control product. The biocontrol fungus colonized several plant species, but only damaged those that were susceptible. Since the biocontrol fungus was applied to soil before plant emergence, colonization of plant tissues likely occurred during the early stages of plant growth and development, but the frequency of fungal isolation decreased with time as the plants continued to grow. Its ability to colonize both resistant and susceptible plant tissues indicated that it has a saprophytic rather than pathogenic nature.

The biocontrol fungus was detected in the soil for a longer time than on the plants, but the populations in soil also declined with time. In the soil, the fungal populations declined by nearly 50% after 38 weeks. At these levels, the biocontrol fungus was unable to cause damage to susceptible host plants because a related study showed that susceptible crops (such as pea and sunflower) may be sown into soils previously treated with the biocontrol fungus (one year following application to the soil) without developing chlorosis or other effects on emergence and plant growth. Declining frequency of isolation and detection by molecular methods from plant tissues and soil over time provided evidence that the fungus does not multiply, reproduce, or persist on target and non-target host plants, and in soil.

It is also unlikely that the biocontrol fungus can move any significant distance horizontally in soil. The fungus was detected at similar levels in the plot margins as in the untreated control plots, and the frequency of detection in these areas declined with time, thereby reducing the risk of spread to neighboring non-target plants. The fungus did not appear to move away from the site of placement under natural means. There was carryover to new areas mainly caused by applicator error. It is unlikely that significant drift occurred after application because little contamination occurred in control plots and outside of the treated margins despite several people walking on the plots and margin areas. There is some evidence that the biocontrol fungus may move vertically in the soil profile over the course of a year. However, most fungi cannot survive for long periods in deeper soil depth due to less oxygen. Further studies may need to be done to determine survival over time at deeper soil depths. These features greatly reduce the risk of the fungus spreading and persisting in the environment.

Two different approaches were used in this study. Similar results obtained by both methods helped to validate the results, despite the limitations with the methods. The

traditional microbiological techniques for isolation of fungi from plants and soil detected fungal viability, as well as relative population numbers. The limitations were in developing a semi-selective medium for identification of the correct fungal species; note it was not possible to know whether the colonies were from the released strain or a natural wildtype contaminant. Using the specific DNA probe, identification of the exact strain was assured, but it was not known whether the DNA came from living or dead propagules. If no fungal DNA was detected then it could result from its absence in the plot, degradation of the DNA by other microorganisms, or possibly failure in the technique. Extracting clean samples of fungal DNA from soil is difficult and soil samples with higher amounts of organic matter can affect the quality of the DNA. Storage of soil samples before extracting DNA resulted in degradation of sample quality.

In conclusion, the biocontrol fungus had limited impact on the environment. The effects were mostly limited to the season of application and the site of placement. The greatest effects were observed at the time of application and for up to 9 weeks. After that time the fungal populations declined, so carryover from season to season was significantly reduced. Care may need to be exercised during application to prevent drift onto to emerging non-target plants nearby the site of application if rates greater than 63 g/m^2 are used.

Table 1. Summary of analysis of variance of the isolation of the biocontrol fungus from plant tissues (leaves, crowns, roots) of weed and nontarget plant species (Canada thistle, barley, lentil) grown in soil infested with the fungus or in untreated soil. Samples for fungal isolation were taken 10, 20, and 30 days after seeding.

	Trial 1			Trial 2		
Source of variation	Degrees of freedom	F-value	Pr > F	Degrees of freedom	F-value	Pr > F
Treatment (T)	1	2.73	NS	1	25.20	0.01
Host (H)	2	0.76	NS	2	6.30	0.02
Plant tissue (P)	2	0.18	NS	2	3.81	0.02
Days (D)	2	4.27	0.02	2	11.51	0.01
Rep	2	1.94	NS	5	0.44	NS
T x H	2	3.75	0.03	2	6.30	0.02
T x P	2	0.23	NS	2	3.81	0.02
T x D	2	1.13	NS	2	11.51	0.01
T x H x D	8	2.18	0.03	8	3.54	0.01

Table 2. Mean percent isolation of the biocontrol fungus from plant tissues of barley, lentil, and Canada thistle grown in soil either untreated or treated with the biocontrol fungus sampled over 30 days.

Trial	Host	Treated	Treated	Treated	Untreated	Untreated	Untreated
		10 days	20 days	30 days	10 days	20 days	30 days
Trial 1	Barley	0	0	44	0	0	22
	Canada thistle	66	11	55	0	0	0
	Lentil	33	0	11	88	0	0
	Pooled SE = 31						
Trial 2	Barley	66	0	16	0	0	0
	Canada thistle	0	0	0	0	0	0
	Lentil	66	16	0	0	0	0
	Pooled SE = 15						

Table 3. Summary of analysis of variance of the number of isolations of the biocontrol fungus from rhizosphere-soil washes of weed and non-target plant species (barley, lentil and Canada thistle) grown in untreated soil or soil infested with the fungus. Samples were taken over a 30 day period.

	Trial 1			Trial 2		
Source of variation	Degrees of freedom	F-value	Pr > F	Degrees of freedom	F-value	Pr > F
Treatment (T)	1	19.66	0.001	1	56.40	0.001
Host (H)	2	2.79	0.001	2	21.91	0.001
Days (D)	2	3.99	0.001	2	12.69	0.001
Rep	2	0.10	NS	5	0.31	NS
T x H	2	2.79	0.001	2	22.24	0.001
T x D	2	3.99	0.001	2	11.48	0.001
T x H x D	4	2.86	0.001	8	3.71	0.001

Table 4. Mean number of colonies of the biocontrol fungus obtained from rhizosphere-soil washes of barley, lentil, and Canada thistle grown in soil either untreated or treated with the biocontrol fungus sampled over 30 days.

Host	Treatment	Trial 1			Trial 2		
		10 days	20 days	30 days	10 days	20 days	30 days
Barley	Treated	0	1.2	0.1	3.5	0.5	0.5
	Untreated	0	0	0	0.2	0	0
Thistle	Treated	5.4	1.2	3.5	0	0	0
	Untreated	0	0	0	0	0	0
Lentil	Treated	12.4	0.5	0.2	7.1	2.9	1.1
	Untreated	0	0	0	0	0	0

Pooled standard error for both Trial 1 and 2 = 1.6

Table 5. Mean number of colonies of the biocontrol fungus obtained from a soil core taken at 30 days from each pot that had grown barley, lentil, and Canada thistle either untreated or treated with the biocontrol fungus in Trial 2. Analysis of variance indicated a significant treatment and crop interaction at $P<0.05$.

Previous Host	Treatment	Trial 2
Barley	Treated	1.7
	Untreated	0
Thistle	Treated	0
	Untreated	0
Lentil	Treated	0.3
	Untreated	0
		Pooled SE = 0.3

Table 6. Summary of analysis of variance on emergence and chlorisis of dandelion in soil previously used to grow barley, lentil, and Canada thistle with or without the biocontrol fungus.

	Trial 1			Trial 2		
Source of variation	Degrees of freedom	F-value	Pr > F	Degrees of freedom	F-value	Pr > F
Emergence						
Treatment (T)	1	11.67	0.02	1	16.17	0.01
Host (H)	2	3.53	NS	2	13.47	0.01
Rep	1	0.54	NS	3	1.50	NS
T x H	2	0.73	NS	2	8.36	0.01
Chlorosis						
Treatment (T)	1	25724.28	0.01	1	12.18	0.01
Host (H)	2	10.26	NS	2	4.70	0.03
Rep	1	164.28	NS	3	1.00	NS
T x H	2	10.26	NS	2	4.70	0.01

Table 7. Mean emergence and percent chlorosis of dandelion in soil previously used to grow barley, lentil, and Canada thistle with or without the biocontrol fungus. Pooled standard error was in Trial 1 and 6.0 in Trial 2.

Previous Host	Treatment	Trial 1		Trial 2	
		Dandelion Emergence	Chlorosis %	Dandelion Emergence	Chlorosis %
Barley	Treated	5.0	88.9	1.3	48.8
	Untreated	10.0	0	10.0	0
Thistle	Treated	0.0	94.5	7.5	0
	Untreated	6.5	0	7.0	0
Lentil	Treated	3.0	94.5	0.0	50.0
	Untreated	5.5	0	3.0	0
		Pooled SE = 1.6	Pooled SE = 5.2	Pooled SE = 2.0	Pooled SE = 6.0

Table 8. PCR sensitivity in detecting the biocontrol fungus from grass roots and soil after inoculation at seven rates of the microbial product under greenhouse conditions.

Rate	Grass root		Soil core	
g/m^2	Rep1	Rep 2	Rep 1	Rep 2
0	-	-	-	-
4	-	-	+	+
8	+	+	+	-
16	+	+	+	+
32	+	+	+	+
63	+	+	+	+
125	-	+	+	+

+ = DNA detected; - = DNA not detected

Table 9. Detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of Dose Effect 2002 (June application).

(9.1) Samples of July 24, 2002

Treatment	Core			12"		24"		Root		
Reps	I	III	IV	I	IV	I	IV	I	III	IV
1000 g/m^2	+	+	+	±	-	±	-	+	+	+
250 g/m^2	+	+	+	-	-	+	-	-	-	-
63 g/m^2	+	+	-	-	-	-	-	+	-	+
0 g/m^2	-	-	-	-	-	-	-	-	-	-

(9.2) Samples of September 25, 2002

Treatment	Core			12"		24"		Root		
Reps	I	III	IV	I	IV	I	IV	I	III	IV
1000 g/m^2	+	+	+	-	-	-	-	-	±	+
250 g/m^2	-	+	+	-	-	-	-	±	-	±
63 g/m^2	-	+	+	-	-	-	-	±	-	±
0 g/m^2	-	-	-	-	-	-	-	-	-	±

(9.3) Samples of May 5, 2003

Treatment	core			12"		24"		Root		
Reps	I	III	IV	I	IV	I	IV	I	III	IV
1000 g/m^2	+	-	+	-	-	-	-	-	-	-
250 g/m^2	+	+	-	-	-	-	-	-	-	+
63 g/m^2	-	+	+	-	-	-	-	-	-	-
0 g/m^2	-	-	-	-	-	-	-	-	-	-

* + = DNA detected; - = DNA not detected; ± = DNA weakly detected

Table 10. Detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of Isolate Effect 2002

(10.1) Samples of July 24, 2002

Treatment	core		12"		24"		Root	
Reps	I	IV	I	IV	I	IV	I	IV
Control	-	-	+	-	+	-	-	-
94-44B	+	+	-	-	±	-	+	+
85-24B	+	+	+	-	+	-	+	-
97-12B	+	+	±	-	±	-	+	+

(10.2) Samples of September 25, 2002

Treatment	core		12"		24"		Root	
Reps	I	IV	I	IV	I	IV	I	IV
Control	-	-	-	-	-	-	-	-
94-44B	+	-	-	-	-	-	-	±
85-24B	+	+	-	-	-	-	±	-
97-12B	+	+	-	-	-	-	±	-

(10.3) Samples of May 5, 2003

Treatment	core		12"		24"		Root	
Reps	I	IV	I	IV	I	IV	I	IV
Control	+	-	-	-	-	-	-	-
94-44B	-	-	-	-	±	-	-	±
85-24B	+	-	-	-	-	-	-	+
97-12B	-	+	-	-	-	-	-	-

+ = DNA detected; - = DNA not detected; ± = DNA weakly detected;

Table 11. Spatial and temporal detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of August Dose Effect 2002.

Treatment	Type	Rep	Weeks after application[⊥]								
			0	1	2	3	4	5	7	9	38
Control	Root	I	*		-	-	±	-	-	-	-
		II			-	-	-	-	-	-	-
	Soil depth 0-3"	I	-	-	-	-	+	-	-	-	-
		II	-	-	-	-	-	-	-	-	-
	Soil depth 4-6"	I						-			-
		II						-			-
	Soil edge-12"	I						-			-
	Soil edge 24"	I						-			-
250 g/m2	Root	I			+	+	+	-	+	-	-
		II			+	-	+	-	-	+	-
	Soil depth 0-3"	I	+	+	+	±	+	+	+	+	-
		II	+	+	+	±	+	-	+	+	-
	Soil depth 4-6"	I						-			+
		II						-			+
	Soil edge-12"	I						-			-
	Soil edge 24"	I						-			-
500 g/m2	Root	I			+	+	+	-	+	+	+
		II			+	+	+	+	+	-	-
	Soil depth 0-3"	I	+	+	+	+	+	+	+	+	-
		II	+	+	+	+	+	+	+	+	-
	Soil depth 4-6"	I						-			+
		II						-			-
	Soil edge-12"	I						-			-
	Soil edge 24"	I						-			+

[⊥]Sample dates were 28/08/02, 04/09/02, 11/09/02, 18/09/02, 25/09/02, 02/10/02, 16/10/02, 30/01/02, 05/05/03

* blank = not sampled; + = DNA detected; - = DNA not detected; ± = DNA weakly detected

Table 12 Detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of Dose Effect 2001.

(12.1) Samples of September 9, 2001

Treatment	core			12"		24"		Root		
And Reps	I	III	IV	I	IV	I	IV	I	III	IV
1000 g/m^2	-	-	-	-	-	-	-	-	-	-
0 g/m^2	-	-	-	-	-	-	-	-	-	-

Moist samples stored at -80C for 13 months before DNA analysis on October 11, 2002.

(12.2) Samples of July 24, 2002

Treatment	core			12"		24"		Root		
And Reps	I	III	IV	I	IV	I	IV	I	III	IV
1000 g/m^2	±	-	±	-	-	-	-	-	-	-
0 g/m^2	-	-	-	-	-	-	-	-	-	-

* + = DNA detected; - = DNA not detected; ± = DNA weakly detected

Personnel:
Dr. C.Y. Chen – Term scientist (RES 01) FTE employed for 4 months;
Dr. Lecong Zhou – NSERC Visiting Fellow in Government Lab, employed for 7 months;
Ms. Cherie Perpelitza – Technician (EG03) employed for 3 months;
Mr. Lasantha Ubayassa – Technician (EG03) employed for 4 months;

Project Developed Materials:
1) DNA-specific probe for biocontrol fungus
2) Poster presentation and written proceedings on CD ROM from the Soils and Crops Workshop 2003, February 17-18, University of Saskatchewan, Extension Division, Saskatoon, SK: Zhou, L., K. Bailey, and J. Derby. 2003. Environmental fate and plant colonization of an indigenous fungus *Phoma macrostoma* effective for biocontrol of broadleaf weeds.
3) Scientific manuscript submitted: Zhou, L., K.L. Bailey, and J. Derby. 2003. Plant colonization and environmental fate of the biocontrol fungus, *Phoma macrostoma*. Biological Control (submitted for review).
4) Scientific manuscript in preparation: Zhou, L., K.L. Bailey, C.Y. Chen, and M. Keri. Molecular and genetic analyses of geographic variation in biocontrol isolates of *Phoma macrostoma*. Mycologia. In preparation).

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1) Financial support from ADF acknowledged in a presentation called "Biosafety issues in the research and development of microbial weed control products in Canada" at the German-Canada Biosafety Research Workshop, October 21-24, 2001, Braunschweig, Germany.
2) Financial support from ADF acknowledged in a presentation called "Biological Weed Control: An alternate pest management solution" at the University of Alberta, December 2001.
3) Financial support from ADF acknowledged in a presentation called "Biopesticides" at the Canadian Government Researchers Forum, ABIC September 15, 2002.
4) Financial support from ADF acknowledged on poster, proceedings, and manuscripts as listed above.

Agriculture and Agri-food Canada / Agriculture et Agroalimentaire Canada

Environmental Fate and Plant Colonization of an Indigenous Fungus *Phoma macrostoma* Effective for Biocontrol of Broadleaf Weeds

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Introduction

- Dandelion, Canada thistle, chickweed and scentless chamomile are common broadleaf weeds in western Canada.
- Two control strategies: Chemical herbicides (effective) vs Biological control (effective + environmentally sound).
- An indigenous fungus, *Phoma macrostoma*, is being developed as biocontrol agent (BCA) to reduce the emergence of these weeds.

Objectives

This research monitored the environmental fate of the BCA:

- host colonization
- persistence in soil
- dispersal in soil

Materials & Methods

- Three fungal isolates: 94-44B, 85-24B & 97-12B
- Greenhouse trial: PCR Sensitivity
- Field trials: Dose, Isolate, Persistence, Temporal & Spatial Monitoring
- Sampling: 1, 2, 4,12 months after application
- DNA extraction: Plants--QIAGEN® Spin Miniprep Kit; Soils-- MO BIO UltraClean™ Soil DNA Kit
- PCR primer design (Fig.1.)
- Experimental design: RCBD, 2-3 replications

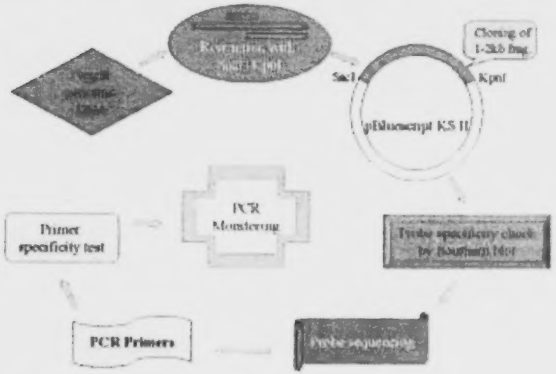

Fig.1. PCR Primer Design for BCA

Results

PCR Dose Sensitivity with *P. macrostoma* 94-44B

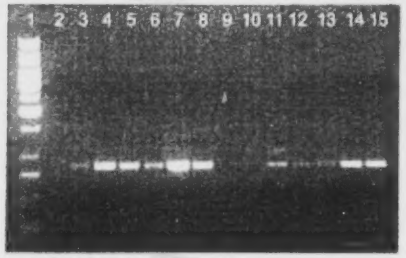

Fig. 2. The inocula were applied at various dosages to glasshouse pots containing field soil. Lane 1: GeneRuler™ 1kb DNA Ladder; Lanes 2-8, upper soil DNA samples extracted from the pots applied with 0, 4, 8, 16, 31, 63, and 125 g/m² of inoculum, respectively; Lanes 9-15, plant DNA templates from the pots applied with 0, 4, 8, 16, 31, 63, and 125 g/m² of inoculum, respectively.

PCR detection of *P. macrostoma* 94-44B applied at various dosages in field conditions

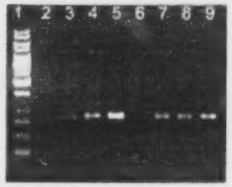

Fig.3. Lane 1, GeneRuler™ 1kb DNA Ladder; Lanes 2-5, Plant DNA samples from the plots applied with 0, 63, 500 and 1000g/m² of inoculum, respectively. Lanes 6-9, upper core soil DNA samples from the plots applied with 0, 63, 500 and 1000g/m² of inoculum, respectively.

Spatial monitoring of *P. macrostoma* isolates under field conditions

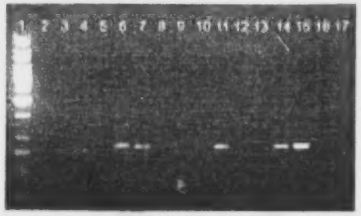

Fig.4. Lane 1, GeneRuler™ 1kb DNA Ladder; Lanes 2-5, PCR using template DNA samples extracted from plant root, upper core soil, 12" and 24" apart soil samples of the untreated control plot. Lanes 6-9, the corresponding samples of the plot treated with 94-44B. Lanes 10-13, the corresponding samples of the plot treated with 85-24B. Lanes 14-17, the corresponding samples of the plot treated with 97-12B. A dose of 500g/m² of inoculum was used for each isolate.

Conclusions

- PCR detection of the BCA was sensitive and the fungus was detectable at a rate as low as 4 g/m² , a dose lower than the recommended application rate of 16 g/m².
- The fungus was detected in soil or plant samples for up to 4 months; after one year, it was not detected.
- The fungus also demonstrated low mobility in soil, since it was not detected horizontally or vertically away from the site of placement.

Temporal monitoring of 85-24B in soil and plants

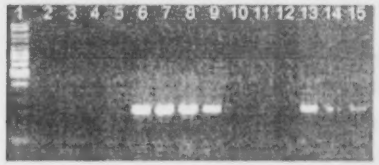

Fig.5. Lane 1, GeneRuler™ 1kb DNA Ladder; Lanes 2-9, PCR using soil DNA templates: Lanes 2-5, DNA isolated from control plots 0, 1, 5, and 9 wk post treatment, respectively; Lanes 6-9, DNA isolated from treated plots 0, 1, 5, and 9 wk post treatment, respectively; Lanes 10-15, PCR using plant DNA templates: Lanes 10-12, DNA isolated from control plots 2, 5, and 9 wk post treatment, respectively; Lanes 13-15, DNA isolated form treated plots 2, 5, and 9 wk post treatment, respectively.

Persistence of *P. macrostoma* in field

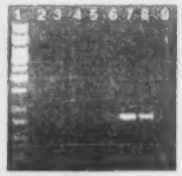

Fig.6. Lane 1, GeneRuler™ 1kb DNA Ladder; Lanes 2-5, PCR using DNA template isolated from soil in untreated control plots 2, 2, 4, and 12 months post treatment, respectively; Lanes 6-9, using DNA isolated from soil in corresponding treated plots.

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